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An Advanced Approach to Simultaneous Monitoring of Multiple Bacteria in Space

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0.0 INTRODUCTION

0.1 Rationale

Crew health is a dominant issue in manned space flight. Microbiological concerns have repeatedly emerged as determinants of flight readiness. For example, in at least one case, suspected contamination of the potable water supply nearly forced a launch delay. In another instance, a crew member's urinary tract infection nearly led to early termination of the mission, partly because of the difficulty of accurately diagnosing the nature of the infection in-flight. Microbial problems are an increasing concern with the trend towards longer-duration missions. It is essential to the success of such missions that systems deliver acceptable quality of air and water during the anticipated lifetime of the spacecraft.

As mission duration and re-supply intervals increase, it will be necessary to rely on advanced life support systems which incorporate both biological and physical-chemical recycling methods for air and water as well as provide food for the crew. It therefore is necessary to develop real-time, robust, in-flight monitoring procedures. In the case of water these procedures should be sensitive enough to detect less than 100 CFU (colony forming units) of bacteria per 100 milliliters. It would also be desirable if the monitoring system could be readily "reprogrammed" to identify specific pathogens if an in-flight incident were to occur.

The overall goal of this project was to develop a microchip-based bacterial monitoring system for detecting and quantifying multiple microorganisms which could be readily miniaturized for in-flight application.

0.2 Background

Traditional methods for identifying and enumerating bacteria are slow and usually overlook numerous organisms because they require cultivation, and also such methods are highly incompatible with the space environment. The development of more effective microbial identification technologies has been a widely sought goal for the past decade. Molecular techniques involving nucleic acids have been particularly promising. The small subunit ribosomal RNAs (16S rRNA in prokaryotes and 18S rRNA in eukaryotes) are an especially promising target for DNA probes because

(1) it occurs in as many as 10,000 copies per bacterial cell, making a preliminary PCR amplification step unnecessary
(2) possesses conserved and hyper-variable regions.

This pattern of sequence conservation makes it possible to design RNA/DNA hybridization probes that can distinguish individual organisms, or groupings of related organisms (Amann *et al.*, 1995; Jurtshuk *et al.*, 1992; & others). Practical diagnostic kits based on this technology (e.g. for *Legionella* and *Chlamydia*) have been successfully introduced into the clinical market.

Specifically the multidisciplinary project involved three components; biology, chemistry, and engineering. The development in each of these program components are presented in the following sections entitled, (1) DNA/RNA Selection and Preparation, (2) Microarray Chemistry and (3) Instrumentation. Feasibility of the microarray-based microbial analyzer was established with "live" water samples taken from the Lunar-Mars Life Support Test Project at JSC. The overall performance of the microbial analyzer exercised on real water samples is described in the Results section. Finally, conclusions are drawn from the project and recommendations provided for additional developments in support of a portable, in-flight microbial analyzer.

1.0 DNA/RNA SELECTION AND PREPARATION

1.1 DNA Probe Design
1.2 RNA Preparation

1.1 DNA Probe Design

Initially several probe targets which would be expected to readily differentiate *Escherichia coli* 16S rRNA from *Vibrio proteolyticus* 16S rRNA in a mixture of both RNAs were designed. Preliminary experiments conducted by the chemistry group suggested that a sandwich assay utilizing both a capture probe and a detector probe would be a particularly promising format. In this scheme a surface capture probe is used to selectively acquire the target rRNA from a total RNA preparation. The detector probe provides a labeling group and assists in the denaturation of the target molecule by disrupting the secondary structure of the target rRNA in the region where it binds to the capture probe. The detector probe should in no instance select against the rRNA of target organisms.

The logical next step was to develop prototype hybridization arrays for detecting several organisms. To insure that this prototype system also has practical utility, additional discussions with the NASA microbiology group were held at Lyndon B. Johnson Space Center to develop a consensus of appropriate organisms to detect. It was concluded that a first generation water quality monitoring system that could simultaneously monitor six microbial groups would be of considerable value. The proposed prototype would: (1) measure total bacterial levels; (2) determine the level of enterics as indicator organisms of fecal contamination; (3) contain specific probes for *E. coli*; (4 and 5) contain genus specific probes for *Enterococcus* and *Burkholderia*; and (6) and contain probes for monitoring the presence of the species *Pseudomonas aeruginosa* and organisms related to it at the genus level.

Initial experiments with pairs of adjacent homologous/mismatched probes to differentiate *E. coli* from *V. proteolyticus* concluded that two mismatches between a probe and a target could be detected in a sandwich assay. All the probes designed herein were therefore required to have at least two mismatches with all the organisms selected against. Three methods were used to design probes depending on the number and nature of both sets of organisms detected and selected against.

In the first approach, sets of aligned sequences were obtained from the public Ribosomal Database Project (RDP) database (http://www.cme.msu.edu/RDP) which include the target species (or genus) and closely related organisms. These sequences were then placed into a sequence editor that allows the sequences to be discriminated according to their identity with the target species. It was then easy to visually identify sequence regions likely to distinguish the target species from other organisms (Figure 1-1). The most promising probes were then searched against the entire 16S rRNA database for spurious matches. This rapid strategy was used to identify the *E. coli*-specific probe pair (Table 1-1).

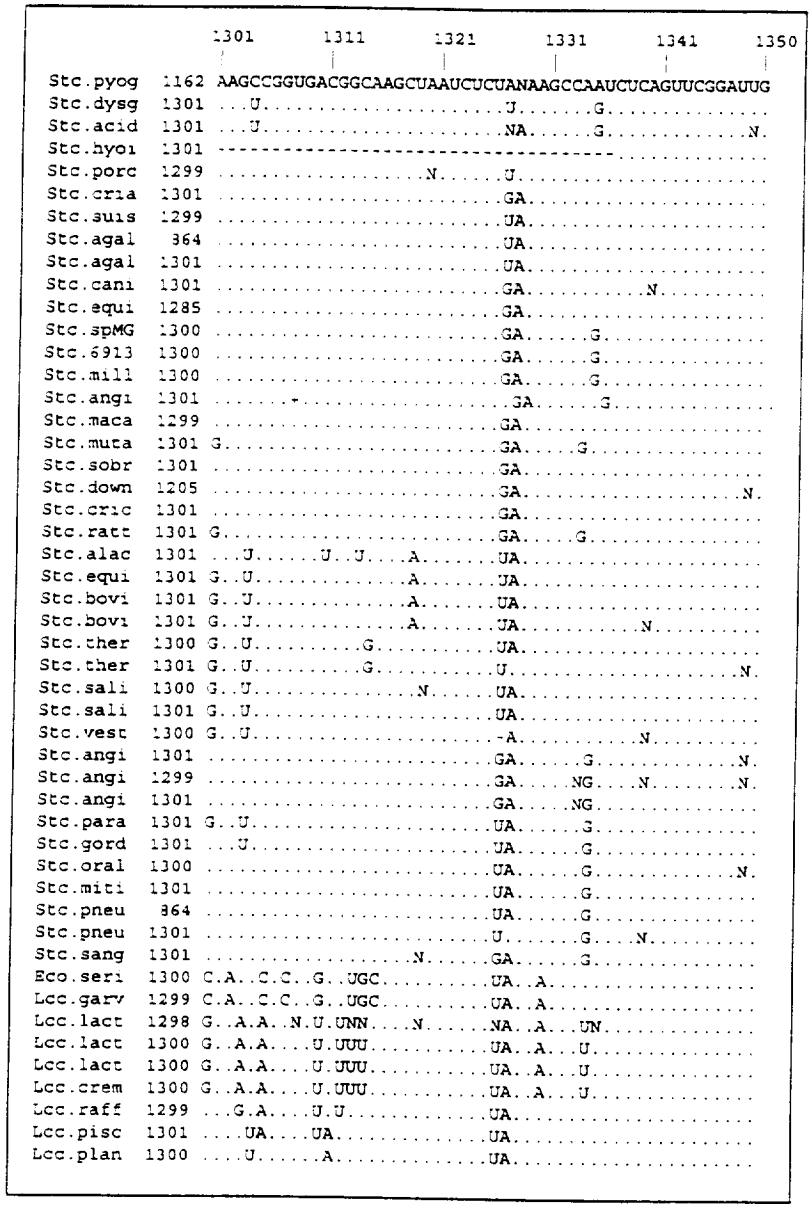
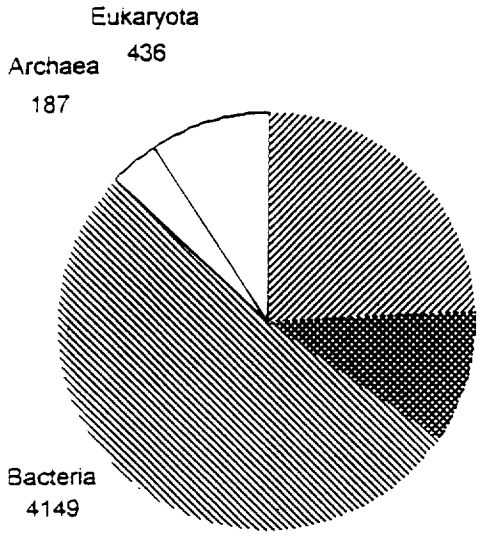


Figure 1-1. Visual of an alignment using the PrettyPrint function in GDE.

This basic strategy was unsuitable to search for probes for all Bacteria, mainly because there is sufficient variability in this data set as a whole, that no single probe of sufficient length binds to all bacteria. Hence, a search for a minimal set of capture probes that would, together, specifically detect all Bacteria (Figure 1-2) was conducted.



⁘ *Bacteria detected by S-D-Bact-0339-a-A-16*
⁒ *Bacteria detected by S-D-Bact-0780-a-A-18*
⁜ *Bacteria* detected by both probes

Figure 1-2. Relative abundance of the available sequences of the three kingdoms in RDP. See Table 1-1 for the probe description.

Since eukaryotic 18S rRNA is very different from prokaryotic 16S rRNA, bacterial probes were sought that would select against archaeal 16S rRNA, and later verified that they did not detect any 18S rRNA. Despite the slight decrease in the number of sequences to manipulate, the number of 16S rRNA sequences in the RDP database was still very large (187 Archaea and 4149 Bacteria), and a computational approach was needed to assist the search for promising probes. Two programs, *find_probe* and *most_mismatch* were designed and coded for this purpose.

A reference organism, for which complete 16S rRNA sequence data is available, was first chosen (e.g., in the case of total Bacteria, or for the enteric probes, *E. coli*). Then, all 13-mers (patterns) from the reference sequence different by at least two bases from all archaeal sequences were identified with *find_probe* (Figure 1-3). The choice of 13-mers was based on the fact that this is the minimal probe length that could be used in hybridization experiments. The patterns were then sorted by increasing number of bacterial sequences in which they were found, using *most_mismatch* (Figure 1-4). The pattern found in the most bacterial sequences was kept as a possible probe target, and the bacterial sequences in which it was not found were extracted in a new file. The file of patterns was sorted again with *most_mismatch* using this new file, and a second target region was then identified. The probe sequences for these two target regions are the complementary sequences from the targets. For each capture probe, a detector probe was chosen adjacent to the capture probes, two bases away. Most detector probes are degenerated at several positions to ensure they bind to all organisms from the target set.

Reference organism: bact.ref
File of target organisms: bact.ref contains 1 organism
File of undesired organisms: archae.pl contains 140 organisms
Maximum number of mismatches allowed with the target organisms: 0
Minimum number of mismatches required with the undesired organisms: 2
Probe length: 13- 13
File name: bact.all
Program started on 02/27/ 98 at 18:36:13 , and finished on 02/27/98 at 21:46:19

Probe	Position(s) in reference organism	Number of target organisms detected
aaauugaagaguu	1,13	1
aauugaagaguuu	2,14	1
auugaagaguuug	3,15	1
uugaagaguuuga	4,16	1
ugaagaguuugau	5,17	1
gaagaguuugauc	6,18	1
aagaguuugauca	7,19	1
agaguuugaucau	8,20	1
gaguuugaucaug	9,21	1
aguuugaucaugg	10,22	1
uuugaucauggcu	12,24	1
uugaucauggcuc	13,25	1
ugaucauggcuca	14,26	1
gaucauggcucag	15,27	1
aucauggcucaga	16,28	1
ucauggcucagau	17,29	1
cauggcucagauu	18,30	1
auggcucagauug	19,31	1
uggcucagauuga	20,32	1
gcucagauugaac	22,34	1
cucagauugaacg	23,35	1
ucagauugaacgc	24,36	1
cagauugaacgcu	25,37	1
agauugaacgcug	26,38	1
auugaacgcuggc	28,40	1
uugaacgcuggcg	29,41	1
gaacgcuggcggc	31,43	1
aacgcuggcggca	32,44	1
acgcuggcggcag	33,45	1
cgcuggcggcagg	34,46	1
gcuggcggcaggc	35,47	1
uggcggcaggccu	37,49	1
ggcggcaggccua	38,50	1
gcggcaggccuaa	39,51	1
cggcaggccuaac	40,52	1
ggcaggccuaaca	41,53	1
gcaggccuaacac	42,54	1
caggccuaacaca	43,55	1
aggccuaacacau	44,56	1
ggccuaacacaug	45,57	1
gccuaacacaugc	46,58	1
ccuaacacaugca	47,59	1
cuaacacaugcaa	48,60	1
uaacacaugcaag	49,61	1

Figure 1-3. First page of the file returned by find_probe with the command line:

>find_probe bact.ref bact.ref archae.pl 0 2 13 13 bact.all

File of probes: bact.all
File of target organisms: bact.des contains 4145 organisms
Number of mismatches allowed with the target organisms: 0
File name: bact.all.sort
Program started at 11:14:53 and finished at 18:22:28

Probe	Position(s) in reference organism	Number of target organisms detected
cagcagugggga	352,364	2149
uguggauuaauuc	950,962	2154
agcagugggaau	353,365	2159
ggcagcagugggg	350,362	2169
auguggauuaauu	949,961	2171
ggaaggugggau	1177,1189	2173
gaggcagcagugg	348,360	2175
aggcagcagugg	349,361	2177
guggauuaauucg	951,963	2186
aagcgugggagc	767,779	2232
auacguucccggg	1375,1387	2256
gggcuacacacgu	1220,1232	2257
gauuacguucccg	1373,1385	2264
aauacguucccgg	1374,1386	2331
acacacgugcuac	1225,1237	2344
cacgugcuacaau	1228,1240	2371
acacgugcuacaa	1227,1239	2374
ggaggcagcagug	347,359	2377
cacacgugcuaca	1226,1238	2383
cgagcgcaacccu	1103,1115	2388
acgugcuacaaug	1229,1241	2393
ugguaguccacgc	798,810	2442
cugguaguccacg	797,809	2463
gacuccuacggga	337,349	2478
auguuggguuuag	1082,1094	2648
cgcaacgagcgca	1098,1110	2697
ccgcaacgagcgc	1097,1109	2722
gggguuaagucccg	1087,1099	2743
gcaacgagcgcaa	1099,1111	2821
guuggguuaaguc	1084,1096	2845
uuggguuaagucc	1085,1097	2847
uguuggguuaagu	1083,1095	2855
caacgagcgcaac	1100,1112	2898
acuccuacgggag	338,350	2996
ccuacgggaggca	341,353	3068
uccuacgggaggc	340,352	3141
cuacgggaggcag	342,354	3146
uacgggaggcagc	343,355	3184
acgagcgcaaccc	1102,1114	3202
aacgagcgcaacc	1101,1113	3205

Figure 1-4. Last page of the file returned by most_mismatch with the command line:

>most_mismatch bact.all bact.des 0 bact.all.sort

Finally, a hybrid of the previous two methods was used to design probes for most genera of Table 1-1. All the 13-mers of a reference sequence different by at least two bases with all other bacteria were identified using *find_probe*. At this point, the sequences of the group are visualized in a sequence editor, e.g. GDE (Genetic Data Environment) and the 13-mers common to the most sequences were further considered and when possible extended to greater lengths.

In some cases the target group is actually comprised of two or more major clusters. Thus, for example, in the case of *Streptococcus*, there are two clusters, "true Streptococci" and *Lactococci*. In this case, after identifying the first target region (for "true Streptococci"), a *Lactococcus* was chosen as a new reference organism and the procedure was repeated for the *Lactococcus* group.

```
                    1301       1311       1321       1331       1341
1350
                    |          |          |          |          |
Stc.pyog  1162 AAGCCGGUGACGGCAAGCUAAUCUCUANAAGCCAAUCUCAGUUCGGAUUG
Stc.dysg  1301 ...U.....................U.......G..............
Stc.acid  1301 ...U.....................NA......G.............N.
Stc.hyoi  1301 ------------------------------------------------
Stc.porc  1299 ..................N.....U.......................
Stc.cria  1301 ..........................GA.....................
Stc.suis  1299 ..........................UA.....................
Stc.agal   864 ..........................UA.....................
Stc.agal  1301 ..........................UA.....................
Stc.cani  1301 ..........................GA..........N..........
Stc.equi  1285 ..........................GA.....................
Stc.spMG  1300 ..........................GA......G..............
Stc.6913  1300 ..........................GA......G..............
Stc.mill  1300 ..........................GA......G..............
Stc.angi  1301 ..........................GA......G..............
Stc.maca  1299 ..........................GA.....................
Stc.muta  1301 G.........................GA.....G..............
Stc.sobr  1301 ..........................GA.....................
Stc.down  1205 ..........................GA.....................N.
Stc.cric  1301 ..........................GA.....................
Stc.ratt  1301 G.........................GA.....G..............
Stc.alac  1301 ...U......U..U....A.......UA.....................
Stc.equi  1301 G..U..............A.......UA.....................
Stc.bovi  1301 G..U..............A.......UA.....................
Stc.bovi  1301 G..U..............A.......UA..........N..........
Stc.ther  1300 G..U..........G...........UA.....................
Stc.ther  1301 G..U..........G...........U.....................N.
Stc.sali  1300 G..U..............N.......UA.....................
Stc.sali  1301 G..U.....................UA.....................
Stc.vest  1300 G..U.....................-A..........N..........
Stc.angi  1301 ..........................GA......G............N.
Stc.angi  1299 ..........................GA.....NG....N.......N.
Stc.angi  1301 ..........................GA.....NG..............
Stc.para  1301 G..U.....................UA......G..............
Stc.gord  1301 ...U.....................UA......G..............
Stc.oral  1300 ..........................UA......G............N.
Stc.miti  1301 ..........................UA......G..............
Stc.pneu   864 ..........................UA......G..............
Stc.pneu  1301 ..........................U.......G....N.........
Stc.sang  1301 ..................N.......GA......G..............
Eco.seri  1300 C.A..C.C..G..UGC..........UA..A..................
Lcc.garv  1299 C.A..C.C..G..UGC..........UA..A..................
Lcc.lact  1298 G..A.A..N.U.UNN....N......NA..A...UN.............
Lcc.lact  1300 G..A.A....U.UUU..........UA..A...U..............
Lcc.lact  1300 G..A.A....U.UUU..........UA..A...U..............
Lcc.crem  1300 G..A.A....U.UUU..........UA..A...U..............
Lcc.raff  1299 ...G.A....U.U............UA.....................
Lcc.pisc  1301 ....UA....UA.............UA.....................
Lcc.plan  1300 ....U......A.............UA.....................
```

Figure 1-5. Streptococcus alignment. Notice that a slightly degenerate probe hybridizing to the 1303-1326 region of S. pyogene would detect almost all Streptococci, but no Lactococci.

These basic procedures were followed in order to design possible capture and detector probes for all six groupings in the prototype water quality monitoring system. The probes are shown in Table 1-1. Some of these probes have been tested in hybridization arrays as described elsewhere in this report.

Table I: Suggested probes and their specificity.

Probe (5' → 3')	Capture for:	Detector for:	Comments
S-D-Bact-0339-a-A-16 cwgcchcccgtaggdg	Bacteria		Degenerate at 3 positions. ≥ 2 mismatches with all Archae Misses S. paucimobilis, X. maltophilia
S-D-Bact-0318-a-A-17 gnccgtrtytcagtncc		Bacteria	Degenerate at 4 positions
S-D-Bact-0780-a-A-18 NH_2-agggtatctaatcctgtt	Bacteria		≥ 2 mismatches with all Archae Misses Staphylococci, some Burkholderiae
S-D-Bact-0801-a-A-17 gtttasngcrtggacta		Bacteria	Degenerate at 3 positions
S-S-E.coli-0466-a-A-17 NH_2-tcaatgagcaaaggtat	*E. coli*		≥ 4 mismatches with others
S-G-Ralstonia-0631-a-A-19 cgtgcagtcaccaatgcaa	Ralstonia		≥ 2 mismatches with all others
S-G-NeisRals-0650-a-A-19 yccctctgacayactckag		Ralstonia and Neisseria	
S-G-Burk-0824-a-A-19 gaatccccaacaactagtt- NH_2	Burkholderia + Ralstonia		≥ 3 mismatches with all others
S-G-Beta-0803-a-A-21 rrcatsgtttagggcgtggac		Burkholderia	Degenerate at 3 positions
S-G-Neis-0632-a-A-17 ayccagttcaraacgca	Neisseria		Misses Vit.setr (2), Crb.viol, Iod.fluv, Kin.oral, Kin.king (2) and Sim.muel
S-G-NeisRals-0650-a-A-19 yccctctgacayactckag		Ralstonia and Neisseria	
S-G-Staph-0460-a-A-17 gaygtgcayagttactt	Staphylococcus [1*]		≥3 mismatches with all others
S-F-Staph-0481-a-A-19 ctttctgrtyagtrccgtc		Staphylococcus group	
S-G-Staph-0226-a-A-22 ctaatacggcgcgggtccatct	Staphylococcus [2*]		≥ 2 mismatches with all others
S---0250-a-A-19 agccgttacctyaccaact		A bit everything	
S-Sb -S.caseolyticus--a-A-20 agcgtcagttrsagaccaga	S. caseolyticus		
S-G-Pseud-0439-a-A-18 NH_2-tgcccttcctcccaactt	Pseudomonas		≥3 mismatches with all others misses: str.B0267, str.BO251, Ps.syring4, Ps.fluore4 (sequence data lacking)
S-G-Pseud-0417-a-A-22 aaagtgctttacaatccgaaga		Pseudomonas	Only P. fluorescens strain MS1650 is undetected.
S-G-Strep-0994-a-A-21 ctargratagcackrgtatgt	Lactococcus		
S-G-Strep-0972-a-A-20 agacctggtaaggttgttgc			
S-G-Strep-1264-a-A-22 agagattwgcytgccgtcaccg	Streptococcus		≥ 2 mismatches with all organisms but Streptococci. Misses all lactococci.
S-P-Gram+-1288-a-A-20 atccgaactgagayyggctt		Gram positive	

S-S-B.cepacia-0456-a-A-17 ggtgtattagagccaa	B. cepacia + Ps. cepacia		≥ 4 mismatches with all others
S-S-R.pickettii-0081-a-A-18 gccatcaatctagcaagc	R. pickettii		Use in conjunction with one of the Burkholderia probes.
S-S-B.mallei-0455-a-A-21 actccgggtattagccagaat	B. pseudomallei + B. mallei		5 mismatches with R.eutrop. ≥6 mismatches with all others.
S-G-Acinetobacter-0436-a-A-19 agccuccuccucgcuuaaa	Acinetobacter		
S-G-Alpha-414-a-A-20 gctttacaaccmwaaggcct		Acinetobacter	Degenerate at 2 positions Misses Can.lwof and Can.anit

[1] this group includes: Stp. sapro3, sapro2, saprop, cohnii, caprae, equorm, haemo3, haemo2, aeroph, homini, homin3, muscae, intmed, schle3, schle2, schlei, warne2, warner, aureu4, aureu5, aureu2, aureus, haemol, arlett, capitc, capitu, capit2, sacly2, epide3, epide5, epider, lugdun, and Spi.spN26

[2] this group includes: : Stp. sapro3, sapro2, saprop, cohnii, caprae, kloosi, equorm, haemo3, haemo2, carnos, auricu, auric3, muscae, sp1, intmed, schle3, schle2, schlei, pulver, sciur3, sciuri, lugdun, and Spi.spN26

- not enough sequence data was available for Stp. auric2, aureu3, saclyt, epide2, and epide4

1.2 RNA Preparation

Before assembling a multiple probe set, it is important to ascertain that each individual probe can distinguish its target group from most likely interfering organisms. Hence, total RNA was extracted from representative strains of the various organisms. Cultures (50 ml) of each bacterial strain of interest were centrifuged at 4°C for 10 min, and resuspended in ACE buffer (10 mM sodium acetate pH 5, 50 mM NaCl, 3 mM EDTA). The cells were lysed by adding first 20 µl of 1 mg/ml lysozyme and incubating 5 min on ice, and then 400 µl of 0.5 M sodium acetate and 200 µl of 10% SDS and incubating 10 min on ice. This was followed by a double ACE-equilibrated phenol extraction, a CH_3Cl:isoamyl alcohol (24:1) extraction, and an ethanol precipitation. The pellets were resuspended in 100 µl H_2O and stored at -20°C. This total RNA was used in hybridization experiments reported in the Results section.

A second aspect of the microbiology effort was devoted to the development of spacecraft-compatible methods of isolating nucleic acids from complex samples (soil, blood, air and waterborne particulates). Of particular interest is methods not requiring the use of toxic substances such as phenol and ethidium bromide, and/or high-speed centrifugation. These constraints eliminate essentially all conventional methods, so the present work has started fresh to lay the groundwork for new approaches.

Selective Adsorption of RNA

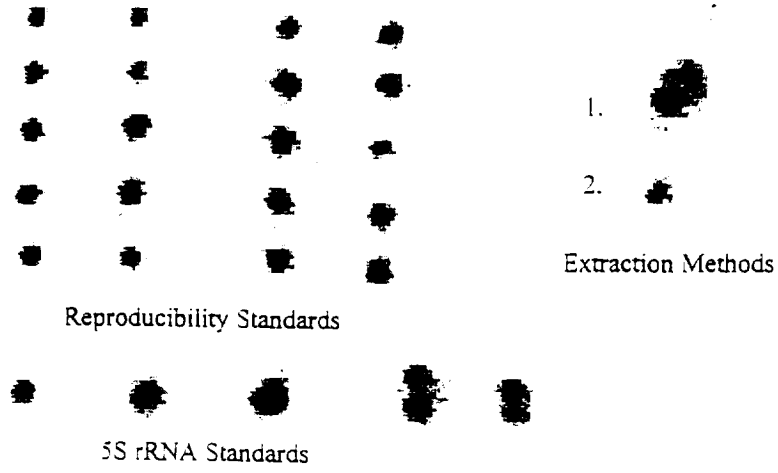
One of the few chemical differences between RNA and DNA which can serve as the basis of separation is the presence of the vicinal 2',3' cis-diol at the 3' end of the RNA molecule. This feature is absent from the deoxyribose backbone of DNA, and is exploited as the basis of recognition by boronate affinity methods. Boronic acid chromatography has been used for tRNA and (ribo)nucleotide isolation since the 1970's (Moore *et al.*, 1974; McCutchan, 1975). Several workers have subsequently

demonstrated the application of boronic acid chromatography to separation of nucleic acids and their derivatives, and of sugars and glycosylated proteins (Ackerman *et al.*, 1979; Benes *et al.*, 1993; Bergold and Scouten, 1983; Bouriotis *et al.*, 1981; Pace and Pace, 1980; Fulton, 1981). In addition, it has been demonstrated that modification of the m-aminophenylboronic acid group to confer a lower pKa on the boronic acid moiety can enhance the range of pH over which these adsorbents are useful (Liu *et al.*, 1994, 1995). We have used batch equilibrium adsorption isotherm measurement and several supporting techniques to characterize the adsorption of mixed RNAs on m-aminophenylboronic agarose under a variety of conditions.

Research indicated that the well-known affinity-enhancing influence of divalent cations depends strongly on the precise nature of the cation used, with barium being far more effective than the conventionally-used magnesium. This adsorption-promoting influence of barium is likely to arise primarily from ionic influences on the structure and rigidity of the RNA molecule, as the adsorption of ribose-based small molecules is not similarly affected. The substitution of barium for the standard magnesium counterion does not greatly promote the adsorption of DNA, implying that the effect is specific to RNA and may be useful in boronate-based RNA separations. RNA adsorption isotherms exhibit a sharp transition as a function of temperature, and this transition occurs at different temperatures with Mg^{2+} and Ba^{2+}. Adsorption affinity and capacity were found to increase markedly at lower temperatures, suggestive of an enthalpically favored interaction process. The stoichiometric displacement parameter, Z, in Ba^{2+} buffer is three times the value in Mg^{2+} buffer, and is close to unity. Consequently, the interest in the use of boronic acid chemistries to capture rRNA for hybridization analysis is warranted.

Also examined was the selective capture of RNA through single-stranded portions in which bases are exposed to selective adsorptive interactions, with the idea that double-stranded DNA and non-nucleic acid contaminants would not be captured by such interactions. Results indicated that, in agreement with our working hypothesis that base nitrogen heterocycles could mediate an interaction with immobilized (chelated) metals, RNA adsorption is favored over that of DNA. Results are being extended through the work on gentle elution, compatible with direct introduction of the semi-purified RNA samples into a chip-based probe assay, ideally as the next step in a closed system.

Finally, phosphorimager storage plate technology was used to test novel purification methods in comparison with existing standard techniques such as the widely-used Tsai method (Figure 1-6). In addition to being spacecraft-compatible, our methods may find spin-off application on Earth because of their potentially greater convenience. Most recently, the use of nucleic acid conformation-modifying compaction agents was explored to facilitate purification by adsorption and by formation of flocs large enough to be separated from RNA by simple coarse filtration, or low-speed centrifugation (Figures 1-7 and 1-8).

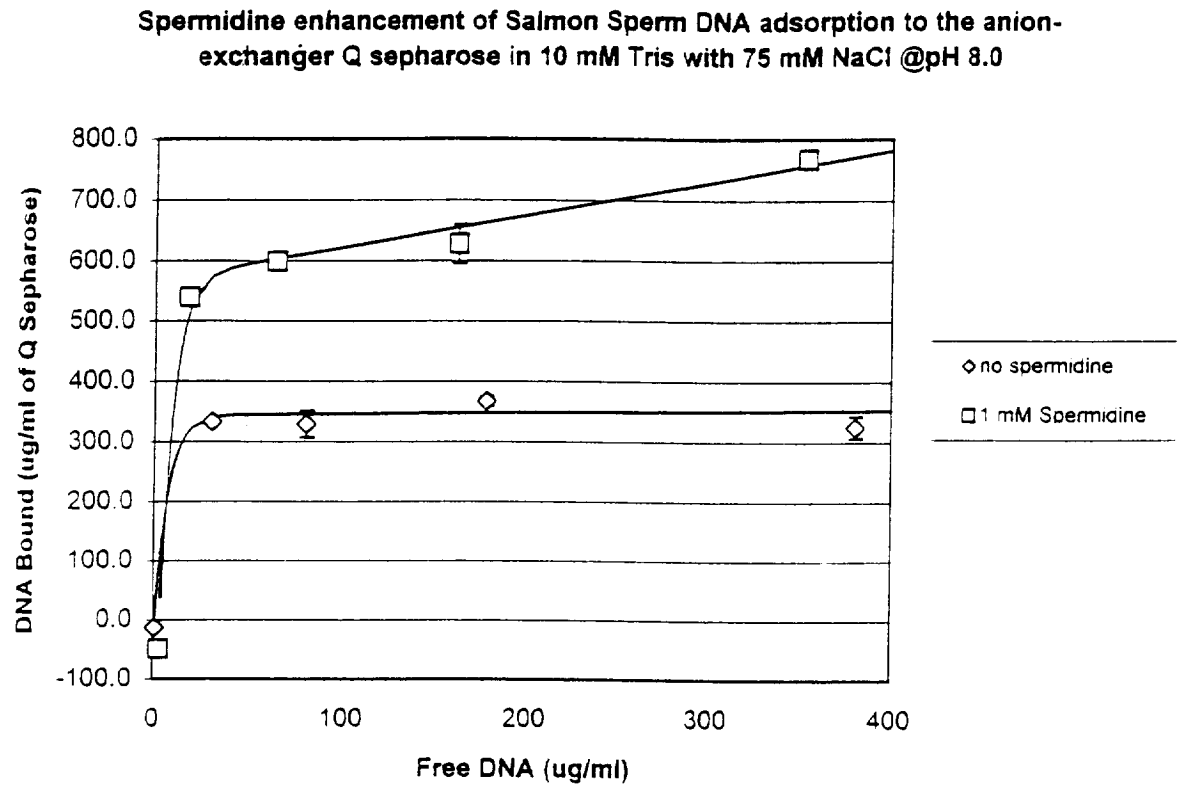


PSL of rRNA purifications between two differing extraction method protocols (far right). Standard controls with known concentrations (bottom).

Spike Recovery Experiment

PSL signals obtained from samples loaded with known, varying numbers of bacteria, after standard rRNA recovery and dot blot hybridization.

Figure 1-6


Spermidine enhancement of Salmon Sperm DNA adsorption to the anion-exchanger Q sepharose in 10 mM Tris with 75 mM NaCl @pH 8.0
DNA Bound (ug/ml of Q Sepharose)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
-100.0
0
100
200
300
400
Free DNA (ug/ml)
no spermidine
1 mM Spermidine

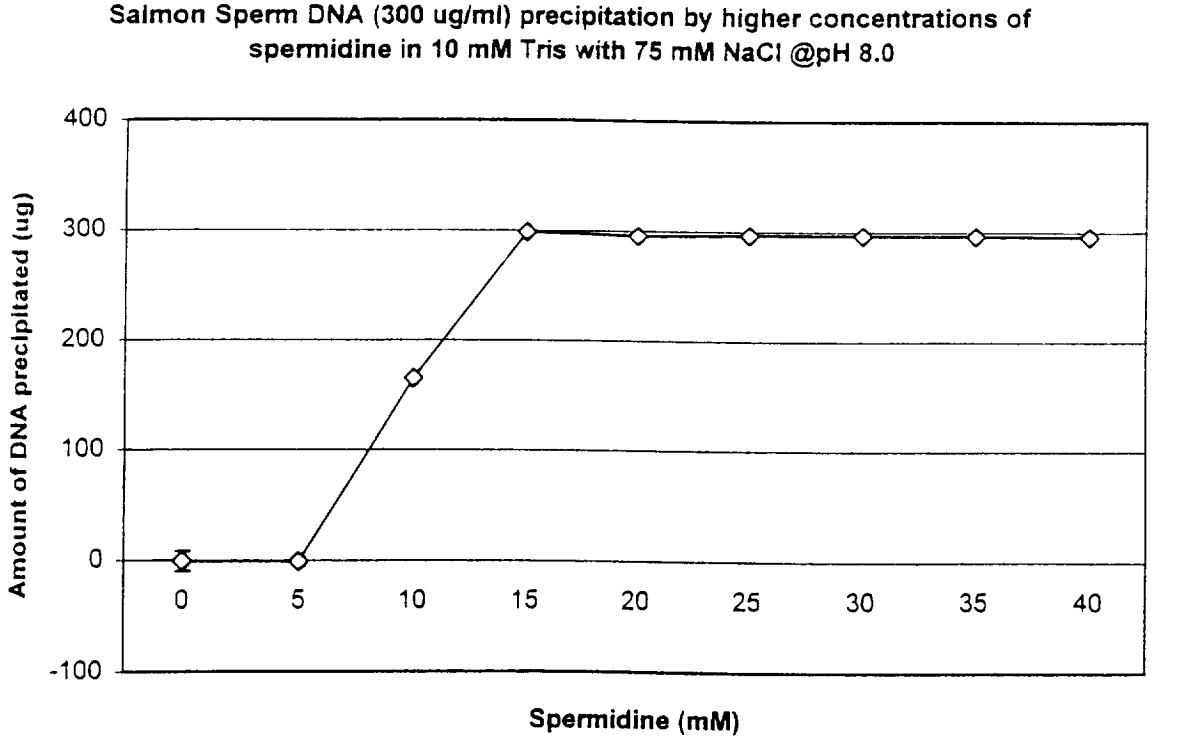

Salmon Sperm DNA (300 ug/ml) precipitation by higher concentrations of spermidine in 10 mM Tris with 75 mM NaCl @pH 8.0
Amount of DNA precipitated (ug)
400
300
200
100
0
-100
0
5
10
15
20
25
30
35
40
Spermidine (mM)

Figure 1-7

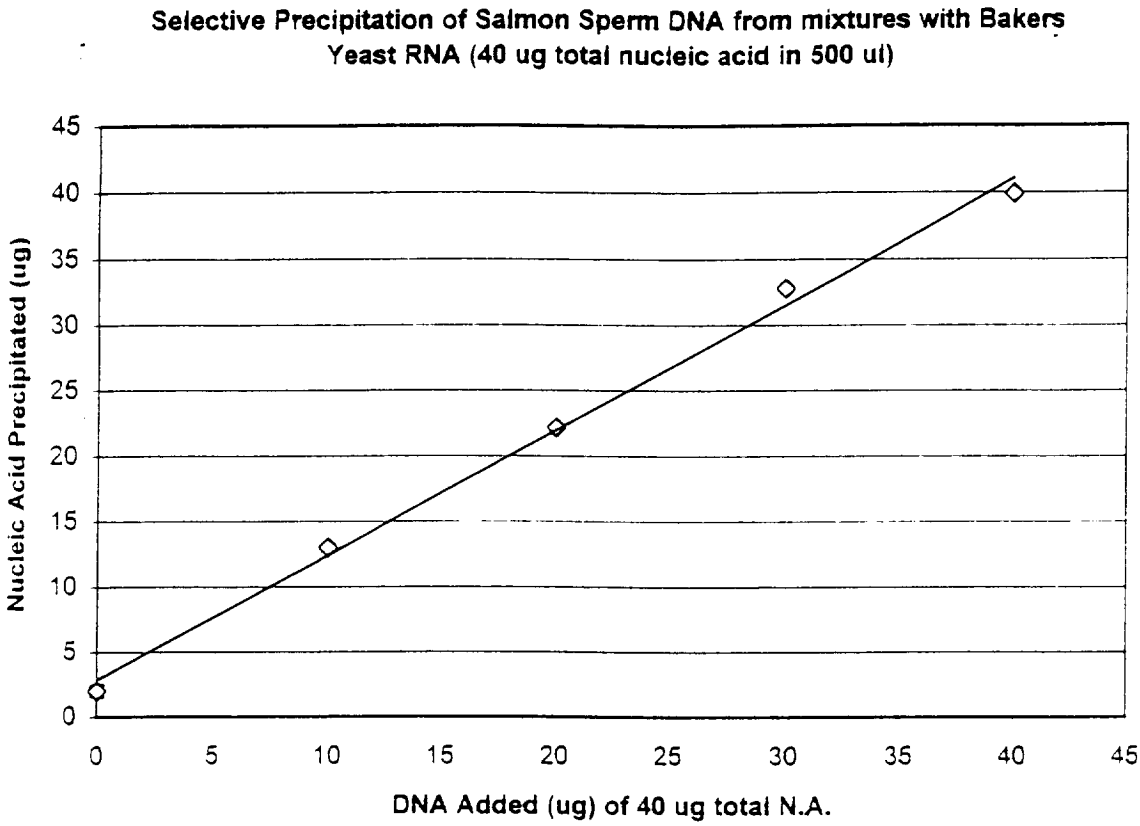

Selective Precipitation of Salmon Sperm DNA from mixtures with Bakers Yeast RNA (40 ug total nucleic acid in 500 ul)
Nucleic Acid Precipitated (ug)
0
5
10
15
20
25
30
35
40
45
DNA Added (ug) of 40 ug total N.A.

Figure 1-8

2.0 MICROARRAY CHEMISTRY

2.1 Synthesis of DNA Probes

A large number of oligonucleotide (short DNA) probes were synthesized with a variety of chemical modifications to serve as 16 S rRNA capture sequences. For modeling studies in the microtiter well format, these probe molecules were synthesized with a terminal biotin group which allowed easy and quantitative coupling to streptavidin derivatized surfaces. In some cases, probes of the same sequence were synthesized as the 2'-O-methyl derivative to compare binding efficiency and specificity. These 2'-O-methyl probes were found to have a somewhat greater affinity for rRNA capture, but at a significant loss of specificity, and were therefore terminated as candidate probe molecules.

Probes were also synthesized with a terminal NH_2 group to allow chemical coupling to epoxide-derivatized glass and other candidate surfaces (such as succinic anhydride activated plastic) which would be compatible with microarray fabrication and proximal CCD imaging. The robotic deposition of these NH_2 modified probes under mildly basic conditions on epoxide-derivatized glass surfaces was found to be both efficient and reproducible, and was therefore employed for all subsequent studies in the microarray format.

A separate class of probes used as solution phase detector probes were synthesized with a terminal digoxigenin group. These probes were found to be effective not only in providing a detectable label on unlabeled rRNA molecules, but also in facilitating target site unwinding and presentation to surface capture probes as discussed below in the hybridization modeling.

2.2 DNA Probe Attachment Chemistry

As mentioned previously, the two primary methods of tethering oligonucleotide probes to solid supports were by the strong streptavidin–biotin interaction in the microtiter well format and by covalent coupling of NH_2 modified molecules on vapor deposited epoxysilane modified glass surfaces. A thorough analysis of probe densities was performed on both types of surfaces. In the case of the streptavidin surface, it was found that the streptavidin modification of wells reproducibly provided essentially a monolayer coverage, and that the probe density converged to a value dictated by the diameter of the streptavidin molecule (~50Å). The binding of biotinylated molecules to such a surface is stoichiometric and essentially irreversible in nature and yielded a density of $5x10^{10}$ molecules/mm^2 under conditions where probe is provided in excess of binding sites.

A similar density of probes was obtained on the epoxide-derivatized glass surfaces when NH_2 containing probes were chemically coupled under mildly basic conditions. In contrast to the streptavidin surface, this chemical coupling is not stoichiometric in nature and requires the addition of

somewhat higher molar amounts of probe to drive the reaction. This does not require a significant expenditure of probes, since, in general, these probes are deposited robotically in a microarray format in picoliter to nanoliter volumes.

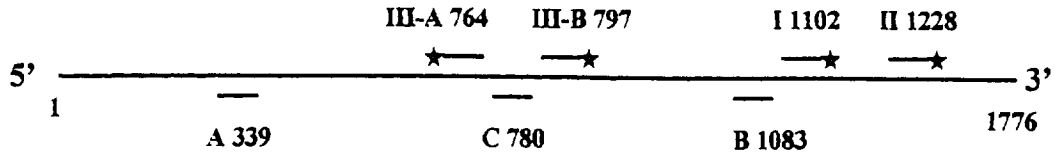
A significant advantage of the epoxide surfaces is the fact that the monolayer provides reactive epoxides which are 5 - 7 Å on center. Since the probe molecules are relatively large and highly negatively charged polyanions, their spacing under ordinary coupling conditions converges to a value of about 50 Å on center due to steric and electrostatic considerations. The importance of this observation is that a large number of epoxides are unused and are available for secondary surface modifications with NH_2 containing small molecules which may have attractive properties with regard to the enhancement of hybridization of 16 S rRNA target molecules. Preliminary results have demonstrated that such modifications are indeed possible, and represent an exciting possibility for the generation of second generation type microbial arrays.

2.3 Hybridization Modeling

A large effort has been made to identify parameters affecting the binding of the relatively large 16 S rRNA molecule to surface immobilized probes. These studies have been performed primarily in the streptavidin modified microtiter well format using chemiluminescent detection of bound molecules. The probes used in these studies have included sequences provided by the University of Houston group as well as some designed by the Baylor group. Targets have included *E. coli* and *Vibrio* intact 16 S molecules, derivatives of these molecules generated by RT-PCR and synthetic targets representing various target sites of interest. Both equilibrium and kinetic data have been acquired under an extremely large set of experimental conditions.

By far, the most significant finding to emerge from these studies is the fact that the secondary structure adopted by the 16 S rRNA molecule in most ordinary hybridization solutions profoundly affects the outcome of surface binding. Although the secondary structure adopted by the 16 S molecule under biological conditions has been well documented, this structure is dependent on the presence of the cellular ionic environment as well as the presence of many ancillary 16 S associated proteins. Thus, this structure does not allow *a priori* knowledge of binding site availability in hybridization buffers lacking these factors. Research indicated that the inclusion of a solution state detector probe which was positioned to bind immediately adjacent to the capture probe could eliminate the apparent secondary structure effect in the target molecule. This effect is most likely due to a general unwinding or denaturation of the target molecule in the region of interest. The outcome of the use of such proximal detector or "chaperone" solution probes is illustrated below.

A.



B.

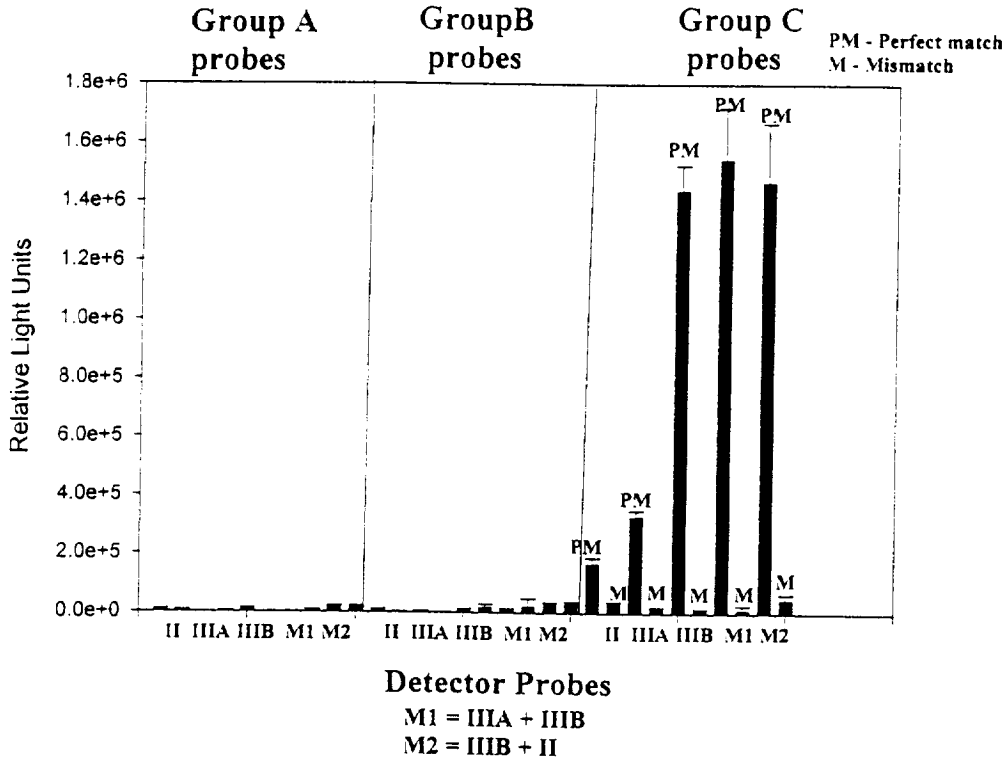


Figure 2-1. Proximal detector probe effect.

In Figure 2-1 A the position of capture and detector probes is shown (detectors above and capture probes below). The sequence of these probes has been previously reported in the July 1997 progress report. Figure 2-1 B shows the result of binding experiments performed with capture probes A, B, and C and detectors II and III. As can be seen from the graph, the strongest hybridization signals are obtained with group C probes and the proximal detectors III-A and III-B. In contrast, Group A and Group B probes do not yield appreciable signal with these detectors or with detector II. These results provide further evidence of the effect of secondary structure on 16 S target binding and the utility of proximally positioned detector probes in overcoming this constraint. Although detector probe I was not synthesized, our prediction is that it

would provide adequate signal with capture probe B (with which it is proximal), and not with probes A and C.

Given these constraints of the secondary structure of large target molecules, investigations are aimed at determining strategies for the hybridization of target molecules in ultra low ionic strength buffers. The use of such buffers would preclude the formation of significant structure of target molecules in solution. In order to provide the requisite screening for capture probe - target phosphate backbone repulsion, surfaces which may be induced to accumulate a positive charge are being tested. In one such scenario, probes are being deposited on a background of a small histidine containing peptide (His_2Glu) whose charge may be modulated from +1 to -1 in the pH range of 5 - 8 (the pK of histidine is 6.5). Preliminary evidence suggest that target molecules may be bound by specific duplex formation in very low ionic strength buffers when this type of surface is positively charged (pH 5). By elevating the pH to 7 - 8 this surface can also contribute to the stringency of hybridization by accumulating a net negative charge. In addition, modified detector probes are being designed which contain small cationic peptides to allow binding of the detector in the same low ionic strength hybridization buffer. In this case, a single detector (for instance, complementary to the bacterial universal probe sequence) may be used for all bacterial 16 S species.

2.4 RNA Labeling Methods

Excellent results may be obtained using an enzyme-linked fluorescent assay (ELF, Molecular Probes) coupled with a proximal CCD detector. In this type of assay, either biotin or digoxigenin may be used as a recognition hapten for the target or detector molecule. Using streptavidin or anti-digoxigenin conjugated alkaline phosphatase, the ELF substrate is cleaved to yield a fluorescent insoluble precipitate which accumulates at the sites of probe addresses which contain bound target molecules. This fluorescent precipitate is stable, does not bleach easily and has excellent spectral properties with regard to the CCD detector (excitation - 337 nm, emission - 542 nm). Using the signal amplification inherent in this type of enzymatic assay, 16 S rRNA target molecules bearing a single digoxigenin-labeled detector have been detected on microarrays at concentrations in the 10^{-13} M range in a volume of 25 µl. In addition, the use of these enzyme conjugate assay schemes allow for the development of other types of substrate development (such as color), which may be of practical importance in limited equipment environments such as in space.

3.0 INSTRUMENTATION

During the course of the program, a new capillary microarray fabrication method was developed and an existing CCD microarray imager was refined as described below. Both systems are amenable to miniaturized, high throughput operation. Specifically the dispensing system is capable of printing a DNA microarray per second, whereas the CCD-based imaging system provides quantitative detection and imaging within a few seconds.

3.1 Microarray Printer

A printer was developed for fabricating DNA microarrays utilizing capillary tubing to dispense extremely small amounts of DNA probe solution (50pL, 50μm spot diameter) onto the interior bottom surface of 96 well microtiter plates, or onto microscope slides as illustrated in Figure 3-1. As shown, a storage vessel which contains the appropriate solutions is pressurized momentarily in order to prime the tubes to initiate the capillary dispensing action. Upon contact with the microtiter plate bottom, the capillary tubes simultaneously deliver small volumes of the DNA solutions at precise locations controlled by the spatial arrangement of the bundled capillaries.

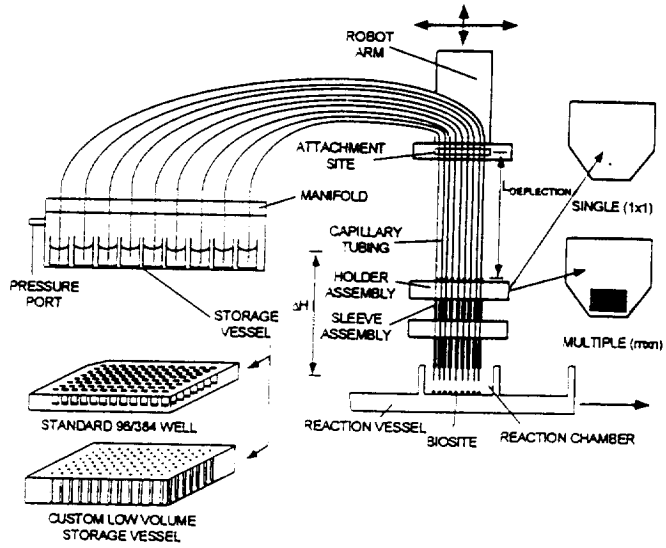


Figure 3-1. Biosite deposition system using multiple capillaries.

In the low volume manufacturing prototype shown in Figure 3-2, 190 micron outer diameter capillaries are threaded through an attachment site at the top of the printing fixture. The tubes extend down from the attachment site through an area allowing the capillaries to flex during printing. Below the flex region the capillaries are threaded through a set of fused silica sleeves held in a grid pattern by the aluminum holder assemblies.

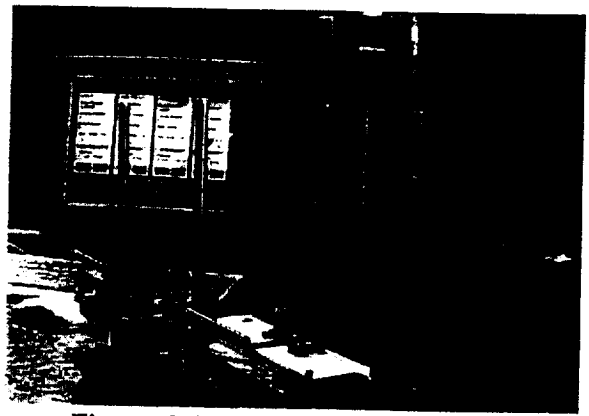


Figure 3-2. Prototype microarray manufacturing system.

The microarray printer proved to be an extremely efficient instrument (1 array/ second print rate) for fabricating medium density microarrays (10-1,000 probes/cm^2). Moreover, Genometrix subsequently built a 4-head version of the printer with internal funds for high throughput commercial fabrication of microarrays within a class 1,000 clean room. The manufacturing capacity is estimated at one million microarrays per year.

3.2 Microarray CCD Imager

The heart of the microarray imaging instrument is the highly sensitive proximity (lensless) CCD imaging technology which provides high throughput detection and imaging of molecules labeled with chemiluminescent or fluorescent reporter groups.

As shown in Figure 3-3, a labeled molecular sample such as a DNA microarray is placed in direct proximity to the CCD sensor. Photoelectrons from the labeled sample are collected and quantified by the instrument within seconds.

The number of photoelectrons generated at each pixel is directly proportional to the number of labeled molecules in such proximity. Consequently, molecular detection and imaging can be quantitatively provided in an automated digital format.

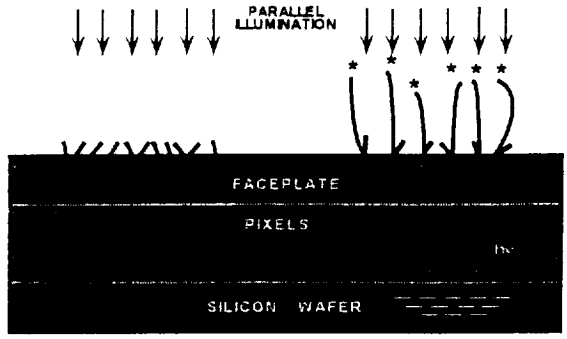


Figure 3-3. CCD proximal detector schematic.

The PC-based CCD proximal detector/ imager shown in Figure 3-4 is approximately 10-fold more sensitive than conventional phosphorimagers and fluor-imagers, and represents a triumph over numerous engineering challenges.

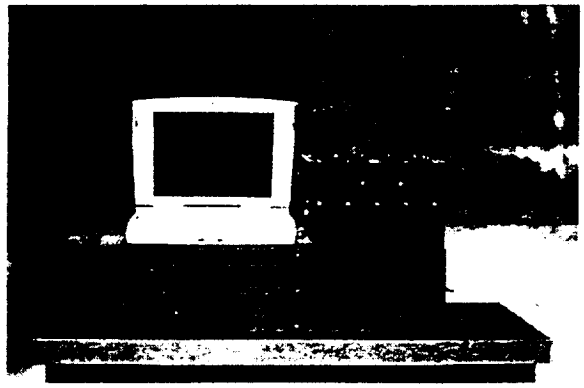


Figure 3-4. PC-based proximal CCD detector.

4.0 RESULTS

In addition to the developments arising from individual program components reported in the previous sections, results are also reported for the integrated microbial detection system tested on *live* waste water samples collected from the Lunar-Mars Life Support Test Project.

4.1 Sample Collection/rRNA Isolation

Environmental waste water samples were collected from the Lunar-Mars Life Support Test Project at JSC. Grab samples were obtained from three separate sample points from the Immobilized Cell Bioreactor System.

Sample point #1 was taken from the Main Feed Tank (TK-Bio-01). Sample #2 was obtained from the TFB Product Tank (TK-Bio-21) and finally sample point #3 was obtained from the ICB Effluent Column. Due to time constraints, only rRNA samples from the Main Feed Tank were analyzed by microarray hybridizations. A schematic of the main feed tank is illustrated in Figure 4-1 below.

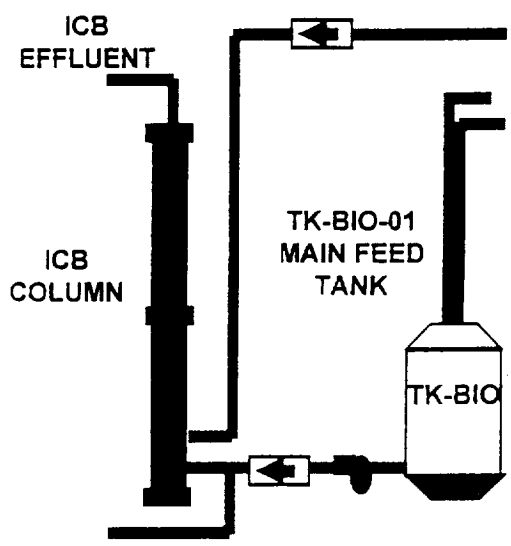


Figure 4-1. Lunar-Mars water treatment system.

Water samples (250 mL each) were centrifuged to pellet the solids and frozen at –20°C until processed for rRNA isolation. All ribosomal RNA preparations were performed using Qiagen's RNeasy Total RNA Purification kit according to the manufacturer's instructions.

Total RNA was isolated from approximately 50 mg of microbial cell paste or sample pellet. After purification, an agarose gel was run to confirm presence or absence and quality of rRNA isolated from the three environmental sample points. A photograph of the gel is shown in Figure 4-2 below.

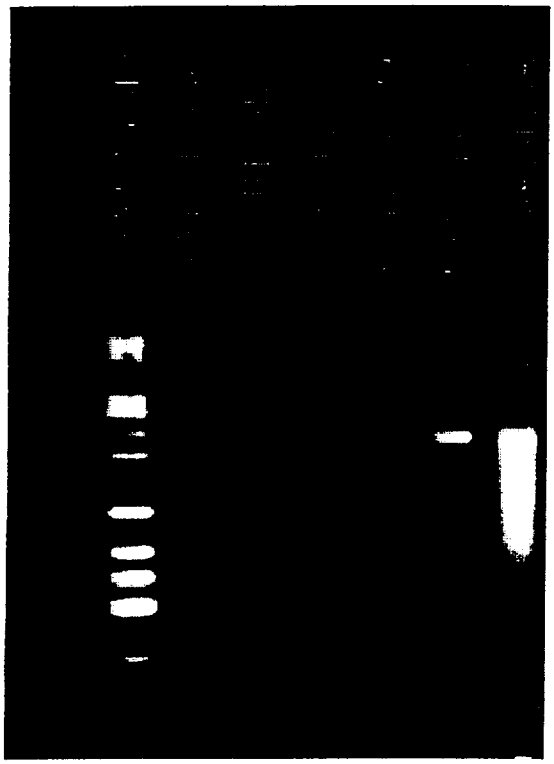


Figure 4-2. Ribosomal RNA isolated from various Lunar-Mars water treatment sample points as well as several known bacterial isolates.

4.2 DNA Probe Selection

Oligonucleotide sequences were designed by George Fox's Lab at the University of Houston. Microbial-specific sequences were chosen by computer alignment of rRNA sequences from an NCBI database. Alignment and probe design were carried out using GCG Software (Genetics Computer Group, Wisconsin Package 9.0).

Species-specific oligonucleotide capture sequences were synthesized using conventional automated DNA phosphoramidite chemistry and purified using HPLC.

Oligonucleotide capture and detection-chaperone sequences are outlined in Table 4-1 and Table 4-2 respectively.

5' –ACTCCGGGTATTAGCCAGAAT 3'
5'- GGCTGTATTAGAGCCAA 3'
5' –GGCTATATTAGAACCAA 3'
5' –GCCATCAATCTAGCAAGC 3'
5' –TATCCCACATCATCCAC 3'
5' –TCAATGAGCAAAGGTAT3'
5' –AGCCTCCTCCTCGCTTAAA 3'
5' –TGCCCTTCCTCCCAACTT 3'
5' –ATTAATCCACATCATCCAC 3'
5' –GAATCCCAACAACTAGTT 3'
5' –CTGCCTCCCGTAGGAG 3'
5' –AGGGTATCTAATCCTGTTT 3'
5' –GGGACTTAACCCAACA 3'

Table 4-1. Microbial microarray oligonucleotide capture sequences.

5'-TTCTTCCGGTACCGTCAT 3'
5'–TTCCGGTACCGTCATCCC 3'
5'–TTCCGGTACCGTCATCCC 3'
5' –TACTCACCCGTTCGCCAC 3'
5' –TTTCGCGTTGCATCGAAT 3'
5' –TGCTTCTTCTGCGGGTAAC 3'
5'- GCTTTACAACC(A/C)(A/T)A AGGCCT 3'
5'- AAAGTGCTTTACAA- TCCGAAGA3'
5' -AGGTT(C/T)TTCGCGTTGCAT 3'
5'- G(N)CCGT(A/G)T(C/T)TCA GT(N)CC 3'
5'-GTTTA(C/G)(N)GC(A/G)TG GACTA 3'
5'-TCAC(A/G)(A/G)CACGAG CTGACG 3'

Table 4-2. Microbial microarray oligonucleotide detector sequences.

The standard hybridization format used throughout the experiments is illustrated in Figure 4-3.

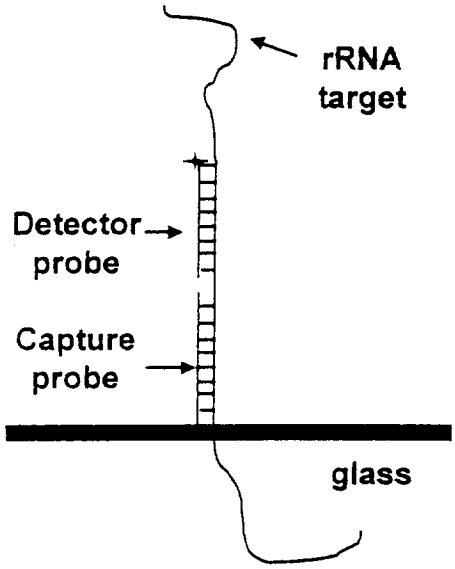


Figure 4-3 . Hybridization format.

Oligonucleotide capture sequences were printed on microslides to form a 4 x 4 microarray using the robotic capillary printing system. The four outside corners of the array were delineated with an immobilized biotin-labeled control marker which also serves as a detection reagent control spot. The array configuration is illustrated in Figure 4-4.

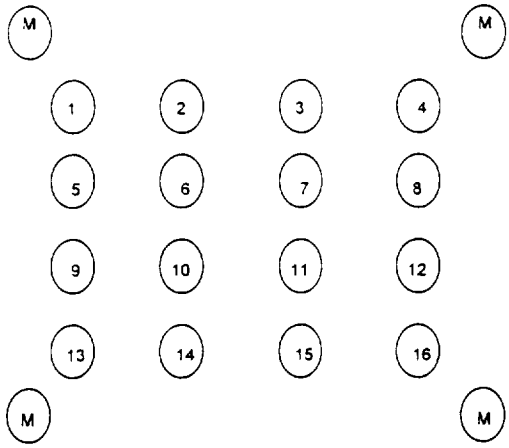


Figure 4-4. Microbial microarray layout.

Key
M-detection marker
1 -Blank
2 -Blank
3 -Pseudomonas
4 -Pan Probe (all bacteria)
5 -Ralstonia Picketti
6 –Staphylococcus 760
7 -Staphylococcus 692
8 -Staphylococcus 629
9 -Burkholderiae 823
10-Burkholderiae Pseudomallei
11-Burkholderiae Cepacia
12-Ralstonia Picketti 942
13-Acinetobacter
14-Escherichia coli
15-Burkholderiae Cepacia (MIR strain)
16-Burkholderiae/Neisseria

4.3 Microarray Fabrication

Glass microslides were purchased from Cell-Line Inc. per Genometrix specification (see Figure 4-5) and washed using an ultrasonic cleaner (Aquasonic Model 410T). Specifically, a formulated cleaning concentrate (Branson GP) was diluted 50:1 in warm tap water. The slides were immersed in the cleaning solution and sonicated for 5 minutes. Following sonication, the slides were rinsed in progressively more deionized and de-mineralized water dip tanks. The final water rinse was made in deionized and glass distilled water of the highest purity.

Following the water wash, the slides were briefly immersed in an acetone bath, followed by a methanol final rinse. The slides were oven dried at 45°C for 15 minutes and chemically derivitized by vacuum deposition of epoxysilane using a

carrier solvent in a conventional vacuum oven at 80°C for 16 hours (at 25" Hg).

Next, the slides were removed from the oven and stored in a clean dry place until printing of capture sequences using the microarray printer.

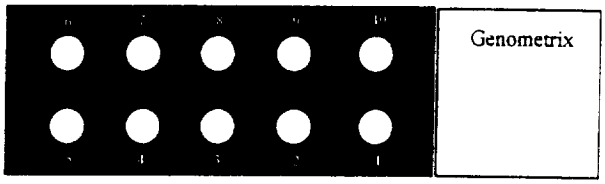


Figure 4-5. Teflon-coated glass microslides.

4.4 Microarray Hybridization

Microarray hybridizations on microslides were performed by a simple three step process as outlined below.

Step 1
Pre-hybridize each well in 100 uL of pre-hyb solution (0.6 x SSC/5X Denhardts) for 20 minutes at room temperature. The pre-hyb solution is removed with a pipetteman immediately followed by step 2 below.

Step 2
To fifty microliters of purified rRNA in 10 mM Tris-HCl (7.5) was added 32 uL DEPC dH20 and 10 uL of 1uM detector oligo mix. Estimated rRNA target concentrations over each array is approximately $1x10^{-8}$ M. The sample is heated for 3 minutes at 72°C and snap chilled on ice. The final hybridization mix is completed by adding 3 uL 20xSSC and 5 uL of 100X Denhardts. A 20 uL aliquot is added to each of three pre-hybridized microarray wells from Step #1 above. Hybridizations are allowed to proceed for 90-120 minutes at room temperature.

Step 3
After the hybridization is complete, the hybridization solution is removed with a pipetteman, followed by three consecutive 100 uL stringency washes at room temperature with 0.6XSSC + 5X Denhardts.

4.5 Microarray Labeling

Microarray labeling proceeded immediately following the stringency washes. Each well was subjected to the following four step process.

Step 1
Each microarray well is rinsed once with 100 uL of conjugate wash buffer (1xTBS+0.1% Tween 20) for 30 seconds.

Step 2
The wash buffer is removed using a pipettman followed by incubating each well in 25 uL of conjugate (1:1000 dilution of a streptavidin- alklaline phosphatase conjugate in wash buffer) for 45 minutes.

Step 3
After removal of the conjugate, each well is washed three additional times with 100 uL of conjugate wash buffer.

Step 4
ELF™ (Enzyme-linked substrate, Molecular Probes, Inc.) substrate is freshly prepared and 25 uL is added to each well. Substrate development is allowed to proceed in a humidity chamber for 30 minutes at 37°C.

4.6 Microorganism Detection

Microarray detection results are shown in Figure 4-6. Each microbial rRNA sample (100 uL) was hybridized to three identical but separate array wells to assess reproducibility.

The results of hybridizing 20 uL of purified (Qiagen) rRNA isolated from Burkholderiae Cepacia species at a final concentration of 1×10^{-8} M is shown in Figure 4-6 (a). Clearly from the array image, positions 12 (Ralstonia Picketti 942) and 16 (Burkholderiae/Neisseria) provide strong signals which is due to sequence similarity among these species. However position 11 (Burkholderiae Cepacia) does not provide a positive signal in any of the images suggesting that the probe for Burkholderiae Cepacia lacks the desired specificity required for accurate detection.

Figure 4-6 (b) is an image of three arrays hybridized to Ralstonia Picketti rRNA. As expected, biosites 5 (Ralsonia Picketti) and 12 (Ralstonia Picketti 942) provide strong positive signals. Also, biosite 16 (Burkholderiae/Neisseria) indicates strong hybridization which is due to sequence similarity.

Finally, Figure 4-6 (c) is a hybridization performed with rRNA isolated from the Main Feed Tank (See Figure 4-1) waste water from the Lunar-Mars Life Support Test Project. The image clearly shows a strong hybridization signal from 3 (Pseudomonas) and 13 (Acinetobacter). Notice that the lesser intense hybridization signals at biosites 6, 7, 8 collectively, represent probes for Staphylococcus. Hence the sample may also contain Staphylococcus due to the triplicate appearance of such hybridizations.

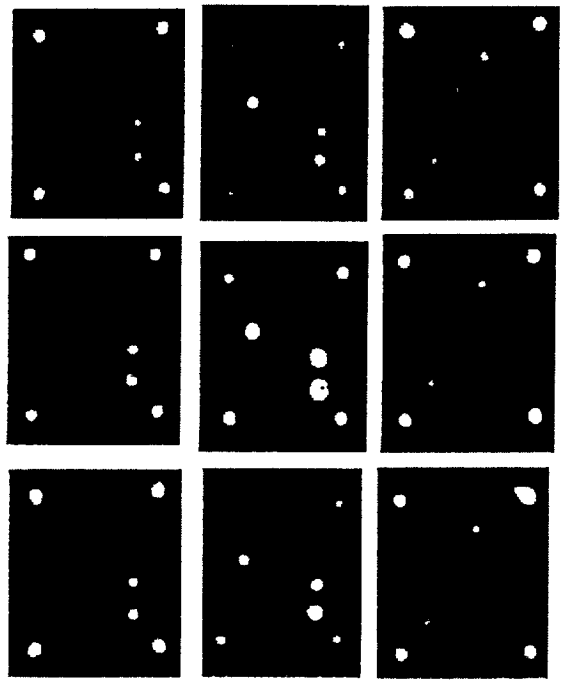


Figure 4-6 . Microbial microarray images.

5.0 CONCLUSIONS

The utility of a novel microarray-based microbial analyzer was demonstrated by the rapid detection, imaging, and identification of a mixture of microorganisms found in a waste water sample from the Lunar-Mars Life Support Test Project through the synergistic combination of:

- judicious RNA probe selection via algorithms developed by University of Houston scientists
- tuned surface chemistries developed by Baylor College of Medicine scientists to facilitate hybridization of rRNA targets to DNA probes under very low salt conditions, thereby minimizing secondary structure
- integration of the microarray printing and detection/imaging instrumentation by Genometrix to complete the quantitative analysis of microorganism mixtures.

Since the presented DNA array-based approach is fast (five minute hybridization with new active surface chemistries followed by 15 seconds for detection, imaging, quantitation, and data storage) and amenable to miniaturization, the results demonstrate the favorable potential for rapid microbial monitoring of air and water supplies.

However, additional issues must be addressed before employment in a space environment, such as safe and efficient methods for collecting and extracting RNA from potentially contaminating microbes. Initial results on the boronate affinity capture methods presented for isolating RNA appear promising. This method offers greater convenience and avoids toxic substances such as phenol and ethidium bromide used on conventional RNA isolation protocols. And finally, all instrumentation components ranging from sample preparation to detection must be integrated into a miniaturized, modular system for ultimate deployment in a space environment.

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